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Many breast tumors progress from estrogen-dependent growth to a more malignant phenotype, characterized by estrogen-independence, antiestrogen resistance, and high metastatic potential. Utilizing 31P NMR spectroscopy, we have investigated this transition utilizing a series of cell lines selected from MCF-7 that reflect characteristics of the progressed phenotype, i.e. hormone-independent (MIII; LCC1), antiestrogen-resistant (LCC2; LY-2), and increased metastatic potential (MIII; LCC1; LCC2). No metabolic changes were found that associated with the metastatic phenotype. However, whilst estrogen treatment produced no consistent spectral changes in any of the cell lines, MIII cells responded to Tamoxifen treatment by consistently increasing several resonances 30%-40% above baseline values. To further understand this finding we are studying the effects of 8-cis-retinoic acid and ICI 182,780, a pure estrogen receptor antagonist. To see the effects of the hormones we are carrying out experiments with cells proliferating in Matrigel, a natural basement membrane,, and we are also testing gas impermeable tubing to assess the effects of oxygen level. We have also written a major article entitled "A History of Biological Applications of NMR" containing over 400 references, for the 50th anniversary of the discovery of the NMR phenomenon.

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Jack S. Cohen 7-28-95

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1. INTRODUCTION

A. Background

Hormonal status is a major factor in the development of breast cancer, and breast cancer is one of the major causes of death in women. We are investigating the metabolism of intact breast cancer cells using MRS methods in order to shed light on the processes involved.

The fact that magnetic resonance techniques have become important in clinical imaging is well known. Perhaps less well appreciated is the role of MRS spectroscopy as a research tool to study the metabolism of isolated intact cells [For those unfamiliar with MRS see Daly and Cohen, 1989]. Such studies can provide important information on biochemical processes, and can be used for the identification of signals and the understanding of metabolic processes in vivo (i.e. the clinical application of MR spectroscopy). Perfused intact cells represent possibly the best approach to the non-invasive study of metabolism. In contrast to the in vivo situation, the cells are homogeneous, particularly when grown in culture conditions. Moreover, they are metabolically stable, and thus much more advantageous to study than suspensions of cells.

Such ex vivo 31P MRS studies have provided information on normal cellular energetic status [Ugurbil et al, 1981; Foxall et al, 1984; Brindle and Krikler, 1985; Painter et al, 1989; Vion-Dury et al, 1989; Miceli et al, 1990], substrate utilization and metabolic pathways [Lyon et al, 1986, 1988, Sri-Pathmannathan et al, 1990], phospholipid pathways [Daly et al, 1987, 1990], intracellular pH changes [Gonzalez-Mendez et al, 1982; Szwergold et al, 1989; Shankar-Narayan et al, 1990], and membrane permeability [Civan et al, 1986]. Using these methods, significant metabolic differences between cell lines have been delineated [Cohen et al, 1986; Kaplan et al, 1990a] and the effects on metabolism following manipulation with nutrients [Gonzalez-Mendez et al, 1982; Karczmar et al, 1983; Desmoulin et al, 1986; Micell et al, 1988; Vion-Dury et al, 1989], hormones [Neeman and Degani, 1989; Galons et al, 1989] drugs [Boyd et al, 1986; Neeman and Degani, 1989; Sonawat et al, 1990; Kaplan et al, 1990b], growth factors [Melner et al, 1983; Kaplan et al, 1990a], and hyperthermia [Suzuki et al, 1989] have been monitored.

A variety of methods using gels as matrices for restraining cells for MRS studies of metabolism are currently available. None of these methods is without flaws, but an appropriate procedure can be adjusted to almost all experimental goals. The most widely used technique is with agarose threads; the advantages of this method are: (a) it is a simple, inexpensive and quick technique; (b) a large number of cells can be maintained in good metabolic status for prolonged experiments (24 to 36 h); (c) the matrix occupies a relatively small volume; (d) effects of metabolite precursors and derivatives, drugs, and physical insults on metabolism can be detected; (e) both anchorage-dependent and -independent cells can be studied.

Detailed studies of cell growth and viability (using trypan blue exclusion and cell counting), microscopy, and the measurement of diffusion constants of metabolites (ATP, glucose) and protein content, were carried out using cells in agarose threads [Foxall and Cohen, 1983; Foxall et al, 1984; Knop et al, 1984; Lyon et al, 1986; Kaplan et al, 1990c]. Agarose is a

carbohydrate gel and in the agarose method cells are simply removed from the threads by gentle pipetting. Matrigel is a natural basement membrane and is a protein gel. It is an extremely open gel that is used extensively for studies of cancer cells [Kleinman et al, 1986, 1993], and even the growth of cells does not alter the diameter of Matrigel threads [Daly et al, 1988]. For Matrigel, dispase is used to dissolve it in order to measure cell densities [Daly et al, 1988]. In fact, these studies taken together constitute perhaps the most extensive documentation of any cell perfusion method that has been used for MRS purposes. It should also be emphasized that a comparison of the same cell line in both the Matrigel cell threads and in tumor xenografts in nude mice gave almost identical spectra [Daly et al, 1988]. From our detailed comparisons and from extensive experience, we have chosen the gel thread method as the most effective one for this work. Therefore, the bulk of this proposal is devoted to studies of perfused intact breast cancer cells embedded in gel threads using 31P MRS.

One of the major challenges in understanding the aberrant functioning of breast cancer cells is in elucidating the complex interactions among such factors as steroid hormones, growth factors, receptors, and cytotoxic drugs [Lippman and Dickson, 1988; Clarke et al, 1989a, 1991]. Since this study was first proposed Tamoxifen has been approved for wide-scale testing as a chemo-preventive agent against breast cancer. In order to understand the role of ER status in breast cancer cells Clarke et al. [1989b], have developed special cell lines, for example the MIII cell line, which has lost the absolute requirement of its parent MCF-7 cells for estrogen, but retains its tumorigenicity [Clarke et al, 1990a]. The cell lines that will be studied in this work are all variants that have been isolated and extensively characterized, and exhibit specific phenotypic changes that reflect critical characteristics of the progressed phenotype, i.e. hormone-independent (MCF-7/MIII; MCF-7/LCC1), antiestrogen-resistant (MCF-7/LCC2; LY-2), and increased metastatic potential (MCF-7/MIII; MCF-7/LCC1; MCF7/LCC2) [Clarke et al, 1989a,b, 1993; Leonessa et al, 1992; Yano et al, 1992; Thompson et al, 1993; Brunner et al, 1993a,b]. The ability to detect specific metabolic changes associated with pheontypic changes is substantially increased, since these cells were all derived from the same parental cell line (MCF7).

It is imperative to study the metabolism of such proliferating cells, and the effects of the anti-estrogen Tamoxifen upon them by MRS methods. Only one other study has been reported before as far as we are aware, on T47D cells (estrogen dependent, equivalent to MCF7) grown on micro-carrier beads [Neeman and Degani, 1989a,b]. An increase in ATP levels was observed on addition of Tamoxifen. However, they used 1 mM concentrations of Tamoxifen, which evidence indicates causes irreversible damage to cell membranes [Clarke et al, 1990b]. One major factor why we believe we may be able to carry out such studies more effectively is the use of Matrigel [Kleinman et al, 1986], in which the cells are proliferating in a natural protein basement membrane gel while being studied by 31P MRS [Daly et al, 1988].

Basement membrane or Matrigel is extracted from a mouse tumor, and is composed of 30% type IV collagen, 60% laminin, 5% nidogen, 3% heparan sulfate proteoglycan, and 1% entacin [Kleinman et al, 1986]. Normal cells grown in basement membrane are morphologically

and functionally similar to their in vivo counterpart, and cancer cells establish a "model tumor", most suitable for metabolic and pharmacological studies. It has been found that the invasiveness of estrogen-dependent MCF-7 cells is increased upon treatment with an anti-estrogen, even though proliferation is inhibited [Thompson et al, 1988]. More recently, absence of estrogen receptors has been correlated with increased basement membrane invasiveness [Thompson et al, 1992]. We would hope to relate our 31P MRS observations on the growth of ER positive and ER negative cells in Matrigel to these phenomena.

B. Specific Aims

- To characterize the growth of several related human breast cancer cell lines in a natural (protein) basement membrane gel (Matrigel) by 31P MRS spectroscopy, and compare the role of estrogen receptor (ER) status in ER positive and ER negative cell lines.
- To observe the effects of estrogen and Tamoxifen on the growth and metabolism of these cell lines with different ER status and hormone dependence, and to investigate the relationship of multi-drug resistance (MDR) to hormone independence in these cell lines.
- To explore the application of 1H MRS spectroscopy to study cancer cell metabolism with water signal suppression and observation of only intracellular signals, and to investigate the 1H signals of choline, creatine, glutamine, alanine and other resolved proton signals in cancer cell spectra, and in response to the agents mentioned above.

2. BODY OF PROPOSAL

A. Cell Perfusion Methods

The use of agarose threads was introduced by Foxall and Cohen [Foxall and Cohen, 1983; Foxall et al, 1984] and is based on the properties of low-temperature gelling agarose (SeaPlaque), that allows mixing of cells with liquid agarose at 37°C, and solidification of the mixture at a lower temperature [for a complete description see Cohen et al, 1989]. 1-1.2 ml of cell pellet ($2 \pm 0.5 \times 10^8$ cells) are mixed with equal volume of 1.8% liquid agarose in phosphate-buffered saline, and immersed in a bath at 37°C for 5-7 min. The mixture is extruded under low pressure through cooled tubing (0.5 mm id) into a 10 mm MRS tube containing growth medium. Using 0.5 mm threads ensures that there is no metabolic compromise, and the cells are viable and in stable energetic status for more than 24 h, while the threads maintain their mechanical strength. Moreover, it was shown that albumin can readily diffuse into the threads [Kaplan et al, 1990a] and thus, the effects of polypeptides and high molecular weight compounds can be studied. The gel threads which fill the tube are concentrated without compression at the bottom of the tube by insertion of a plastic insert with the perfusion fittings. The inflow tube, made of Teflon, is 0.5 mm id, and is placed near the bottom of the tube. The outflow is directed into

openings in the insert, and then into an outflow tube. Perfusion rates (0.3-2 ml/min) are maintained by a peristaltic pump, and since the Teflon tubes are permeable to air, it is not required to include a gas exchanger in the perfusion system. Perfusate can be recycled through a pH-stat when 13C or scarce materials are perfused, or can be wasted, and the cells are then continuously perfused with fresh medium. A debubbler is inserted into the apparatus prior to the insert to remove air bubbles, which may spoil magnetic field homogeneity and affect perfusate flow. In general, the perfusion solution should be the buffered growth medium that is most appropriate for the cells studied.

Cells are routinely perfused for periods from 2 to 12 hours, and sometimes much longer if sterility can be maintained [see publications from Cohen et al, 1986, Daly et al, 1988, Berghmans et al, 1992]. The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that sometimes occurs after ca. 12-24 hours. An initial high level of Pi is indicative of a bad cell sample. If a significant increase of Pi is seen in the first two hours the experiment is usually abandoned. If the Pi/ATP ratio is low, and remains low for this period, the cell sample is considered acceptable (a great deal of experience shows that this parameter is consistent with, but preferable to the exclusion of trypan blue as a measure of cell viability). No other change has ever been seen to occur as long as the cells are adequately perfused. If the perfusion is stopped a rapid increase in the Pi/ATP ratio occurs [Knop et al, 1984]. In order to confirm the adequacy of perfusion in any given case the perfusion rate is routinely adjusted to check for no change in the Pi/ATP ratio (rates vary from 2-0.3 ml/min depending on the circumstances).

Questions have been raised from time to time regarding perfusion of nutrients and oxygen in these threads and into these cells. Detailed quantitative studies of perfusion of ATP [using 31P MRS; Foxall et al, 1984] and of glucose [using 13C MRS; Lyon et al, 1986] were published prior to extensive studies of cell metabolism. It should be noted that these studies explicitly included cells in the agarose gels [Lyon et al, 1986]. Experiments were carried out to induce anoxia in the cells [Cohen et al, unpublished results]. After days of bubbling nitrogen gas through the perfusate this was found to be impossible. The reason was found in the permeability of the fine perfusion capillaries (0.5 mm i.d. polyethylene and similar plastics). Thus the question of reduced oxygen availability does not arise in these perfusion studies for these cancer cells, that need low oxygen tension to survive, and which are mainly glycolytic in any case. This is confirmed by the extreme sensitivity of the cell metabolism as evidenced by their ATP levels to reduced flow (see above). Also, these gels are simply no barrier to oxygen perfusion compared to real tissue for example [Kleinman, 1993]. In perfusion experiments on 14C-labeled albumin (MW 68,500) it was shown that even this large protein diffuses readily through the agarose threads [Kaplan et al, 1990]. Note that in the case of Matrigel, this gel is extremely open [Kleinman et al, 1986], and even the growth of cells does not alter the diameter of the threads [Daly et al, 1988]. In order to study the effects of anoxia on cell metabolism it is necessary to use gas impermeable tubing. These experiments are being performed as the funds permit. A double perspex housing was fabricated to house two oxygen micro-electrodes (from

Microelectrodes, Inc., New Hampshire) for this purpose. A system was previously constructed using capillaries to connect to the perfusion system immediately prior to and after the MRS tube containing the cells. These experiments will be performed with both forms of gel threads (agarose and Matrigel) and at different flow rates, to assess the oxygen consumption of the cells. Different numbers of cells will be used, and appropriate controls will be done (with no cells and dead cells).

One of the disadvantages of perfusion studies with agarose threads is the limited proliferative activity inside the threads. The gel thread technique was therefore improved by the use of a basement membrane matrix, Matrigel, in which anchorage-dependent cells can multiply while being perfused [Daly et al, 1988]. Cell pellet (0.1 ml) is mixed with 2 ml of liquid basement membrane, and the mixture is extruded, as described above for agarose threads, into petri dishes. Cells are allowed to grow in the incubator until the desired densities are reached, and are then transferred to a 10 mm MRS tube. The modification of the perfusion apparatus from one which is used for the agarose threads procedure, includes the insertion of large capacity filters (20 liter) between the peristaltic pump and the tube, which ensure sterility. Thus, the cells can be perfused with fresh medium for prolonged periods (weeks), at a low perfusion rate of 0.5 ml/min, and MRS spectral changes associated with proliferation can be monitored [Daly et al, 1988].

B. Proton MRS Cell Studies

Although much of the initial metabolic work with cells has been done with 31P MRS, proton MRS may play an important role in MRS of intact cells, mainly due to the inherent high sensitivity and universality of this nucleus. This should allow acquisition of data with sufficient signal to noise within a minute, thus enabling the monitoring of metabolic processes on this time scale. However, due to technical difficulties in suppressing the dominating water line and the presence of strong extracellular signals, the potential of 1H MR has been less realized than that of 31P MR.

There are very few significant 1H MRS studies of cancer cell metabolism because of the technical difficulties involved due to the presence of the large water signal and in observing intracellular metabolites against the background of extracellular metabolites. This is a situation that should be overcome since there is potentially a great deal of information contained in the concentration dependence of the proton signals of cells. We recently succeeded in obtaining clean 1H MRS spectra of only intracellular metabolites from perfused MCF-7 cells [van Zijl et al, 1991], and hence intend to explore the possibilities opened up by this novel result.

Major problems encountered in proton MRS are suppression of the abundant water line in order to observe metabolites that are present in mM concentrations and separation of intra and extracellular metabolites. We developed a method to completely separate intracellular and extracellular information in MRS spectra of perfused cells using gradient technology [van Zijl et al, 1991]. The technique uses diffusion weighting to exploit differences in motional properties

between intra- and extra-cellular constituents [Andrasko, et al, 1976]. The MRS signal intensity is a function of the diffusion-weighting factor, which is determined by magnetic field gradients in the sequence. In 1H MRS, both extracellular water and metabolites (diffusion) and intracellular water (selective gradient dispersion [Moonen and van Zijl, 1990]) can be completely suppressed, providing clean spectra of intracellular metabolites [van Zijl et al, 1991]. A total water suppression factor of about 106 is attained. The new method allows monitoring of intracellular metabolism, and of transport of small drugs and nutrients through the cell membrane, under controlled physiological conditions and on a time scale of minutes. Proton NMR studies will be started in the second year of the award, once the conditions for growth in the NMR spectrometer have been established for cells grown in Matrigel and the presence of hormone effectors.

For the proton MRS studies a re-designed perfusion system has been fabricated. This is inserted into the 5 mm MRS probe of the spectrometer and allows perfusate to flow from the bottom to the top. We intend to perform two types of experiments to monitor cell metabolism of viable perfused cells by means of proton MRS: 1) Diffusion-weighted spectroscopy to obtain clean intracellular metabolite spectra, which will be monitored as a function of time after induction of metabolic changes. Particular attention will be focused on the choline (phosphocholine), creatine (phosphocreatine), lactate, glutamine, alanine and other amino acid peaks, in several cell lines. Attempts will be made to resolve diacylglycerol peaks. Several effectors will be added to the perfusate to observe their effects, including Tamoxifen. 2) Gradient-enhanced proton-detected heteronuclear correlation spectroscopy to monitor 13C-glucose metabolism with proton sensitivity [van Zijl et al, 1993].

C. Preliminary Results

(i) Perfusion tubing gas permeability: Initial studies on the gas permeabilities of Teflon tubing have been performed using 2 flow-through oxygen electrodes and oxygen saturated water. The electrodes were first zeroed by immersion in water from which oxygen had been depleted by vigorous bubbling of nitrogen for 20 min. The electrodes were then connected in series by a 5 cm section of 24 gauge Teflon tubing. Water was saturated with 5% CO2 / 95% O2 by vigorous bubbling for 30 min. The oxygenated water was delivered to the flow-through electrodes at a rate of approximately 0.75 mL/min (a typical perfusion flow rate) by peristaltic pump. Both electrodes were calibrated to read 100% at the delivered oxygen saturated levels. A length of Teflon tubing (8 meters) was then placed between the electrodes. This length was chosen as a typical length used for delivery of media to the NMR perfusion tube. The perfusion of the oxygen saturated water was continued for an additional 30 min to allow a steady-state to be reached. Two types of Teflon tubing was tested, a TFF type and a TFP type (both 24 gauge by Atlantic Tubing Company). Preliminary results showed that saturation levels were at 74% of there initial state by the time water had been delivered the length of the TFF tubing. TFP tubing

was less permeable by retaining 84% of the saturation level. The TFP tubing was chosen for use in all current Matrigel thread studies.

(ii) Baseline spectra of cells: A comparison of the properties of the cell lines to be studied in this work are shown in Table 1. Baseline 31P MR spectra of cells lines of interest to this study have now been obtained in perfused agarose gel threads [Ruiz-Cabello et al, 1993]. Cells were routinely perfused for periods from 2 to 12 hours [Cohen et al, 1986; Berghmans et al, 1992]. The only effect that is observed once a steady state is obtained is a gradual loss of ATP and a gradual increase of Pi that sometimes occurs after ca. 12-24 hours. An initial high level of Pi is indicative of an unacceptable cell sample. If a significant increase of Pi is seen in the first two hours the experiment is usually abandoned. If the Pi/ATP ratio is low, and remains low for this period, the cell sample is considered acceptable (a great deal of experience shows that this parameter is consistent with, but preferable to the exclusion of trypan blue as a measure of cell viability). No other change has ever been seen to occur as long as the cells are adequately perfused. If the perfusion is stopped a rapid increase in the Pi/ATP ratio occurs [Knop et al, 1984]. In order to confirm the adequacy of perfusion in any given case the perfusion rate is routinely adjusted to check for no change in the Pi/ATP ratio (rates vary from 2-0.3 ml/min depending on the conditions). In this way baseline spectra of nine cell lines have been obtained. These experiments have been repeated multiple times (3 -5) with consistent results. Although there are relatively few major differences in these baseline spectra, preliminary analysis associate higher levels of PDE and UDPG and lower PC/GPC and PC/PE ratios with the acquisition of hormone independent status. (For further analysis of this data see Ruiz-Cabello et al, 1993). Note that these studies have not yet been performed with the cells in Matrigel.

Table 1: Phenotypes of cells used.

Cell Line	ER Dependence	[1]Estrogen Responsivity	[2]Estrogen Responsivity	TAM Responsivity	ICI 182,780	Metastases
MCF-7	+ ve	dependent	responsive	sensitive	sensitive	no
MCF7/MIII	+ ve	independent	responsive	sensitive	sensitive	yes
MCF7/LCC2	+ ve	independent	responsive	resistant	sensitive	ND[3]
MCF7/LY2	+ ve	responsive	resistant	resistant	resistant	NT[4]
MDA-MB231	- ve	independent	unresponsive	resistant	resistant	yes
MDA-MB435	- ve	independent	unresponsive	resistant	resistant	yes

1 = requirement for E2 to form tumors in nude mice; 2 = respond to E2 by inducing specific genes/mitogenesis; 3 = no data; 4 = non-tumorigenic.

(iii) Hormonal effects: Due to their growth characteristics, the MIII cells provide a unique system for studying the early events associated with progression from hormone-dependent to

hormone-independent growth and invasiveness in human breast cancer [Leonessa et al, 1993]. MIII cells express similar levels of ER to the parent cells [Clarke et al, 1989a], and retain sensitivity to anti-estrogens, e.g. Tamoxifen [Clarke, et al, 1989b]. We have obtained preliminary 31P MRS data [Ruiz-Cabello et al, 1993] demonstrating an unexpected significant increase (ca. 40%) in the ATP, Pi, PME, and in fact most of the phosphate metabolite concentrations, when the MIII cells are treated with Tamoxifen, as opposed to little or no change in the estrogen dependent MCF-7 cells and estrogen receptor negative MDA-MB-231 controls, and lack of an effect on MIII cells when perfused with estrogen. These experiments have been repeated multiple times with consistent results. The data was obtained on cells cast in agarose threads and perfused for 1-2 hours to obtain baseline spectra, followed by addition of 0.5 mM Tamoxifen to the perfusion media. The cells were then perfused for 20-24 hours. The MIII cells were grown in estrogen-free medium without phenol red dye, which has estrogen-like activity. The MDA-MB-231 cells were also grown in estrogen-free medium, but the MCF-7 cells were grown in normal medium. While we do not have an explanation of the surprising finding of the increase in metabolite concentrations in MIII cells, it may reflect a more differentiated or apoptotic phenotype [Armstrong et al, 1992], or an attempt by the cells to reverse the inhibitory effects of Tamoxifen. These preliminary studies show that we can obtain 31P spectra of these cell lines upon addition of hormonal factors. We will do parallel studies on cell growth and MRS in Matrigel in order to optimize these conditions.

(iv) Matrigel perfusion: The use of Matrigel in MRS studies of proliferating cancer cells was developed in this laboratory [Daly et al, 1987, 1988], and was first used in studies of phospholipid pathways in human cancer cells [Daly et al, 1990] (discussed above). The growth rate in the Matrigel was similar to that of in vivo tumors, and concomitant elevations of phosphorus MRS signals were measured. When the cell density increased and neared confluency, the PME/ATP ratio decreased, in agreement with previous data that PME peaks are intermediates of phospholipid synthesis, and decrease towards confluency [Daly et al, 1987]. Also, the chemical shift of the intracellular Pi signal showed that the pH of the cells was becoming acidotic, and this can be used as an indicator of tumor outgrowing.

Matrigel cells threads are currently being produced in a slight modification of the method of Daly et al (1987). Cells are harvested from culture flasks using trypsin and washed with perfusion media. Currently the perfusion media is a modified IMEM containing 5% charcoal stripped fetal bovine serum (CSS) and penicillin/streptomycin antibiotics. We feel that use of antibiotics is necessary due to the rigorous handling of the cells in the gel casting procedure. The cells (approximately 10×10^{-6}) are washed and pelleted in a 15 mL conical tube. Matrigel is added to a total volume of approximately 4 mL and the cells are resuspended. All work is currently being performed in a tissue culture hood in the laboratory. Matrigel, cell pellets, and pipettes are kept in an ice bucket in the hood. The Matrigel/cell suspension is kept on ice while aliquots for threads are removed. The thread forming apparatus consists of a 10 cc Luer tip syringe with a 22 gauge needle. A 60 cm length of 24 gauge Teflon tubing is connected to the

syringe needle. The syringe and tubing are cleaned with 70% EtOH solution before and after use. To produce the threads, an aliquot of the Matrigel/cell suspension is drawn into the Teflon tubing to fill the entire length. The tubing is held, in the hood at room temperature for approximately 30 sec. to allow the suspension to warm. The open end of the tubing is placed into a 100 mm tissue culture dish containing 25 mL of IMEM/CSS media. The solidified thread is extruded from the tubing into the media. Extra threads are singly placed into separate culture dishes to obtain cell counts at appropriate time points.

The extruded threads can be pipetted into the 10 mm NMR perfusion tube by removing the entire contents of a thread-containing culture dish with a 25 mL disposable pipette and allowing the threads to settle to the tip of the pipette over 1-2 min. The threads are then expelled into the NMR tube. Cell counts are performed daily by digesting the singly cultured threads by the addition of 50 mg neutral type II dispase (Boeringer Mannheim) to the 25 mL media and digestion for 10 hr. Thread digests are performed in duplicate. The liberated cells are then counted by hemocytometer and an estimate of the total cell number is made by multiplying the cell counts by the number of threads placed in the NMR tube.

NMR experiments have been performed on MCF7 cells in Matrigel threads perfused with estrogen (1 nM) containing IMEM/CSS media. A pulse-width experiment was first performed to optimize the detection of the β-ATP peak. The β-ATP peak is used since it is not convolved with other phosphorus containing biomolecules. Based on these experiments, a pulse width of 60 ms was chosen. A spectral series of hormone dependent MCF7 cells proliferating in Matrigel over 3 days is given in Figure 1. The effects of proliferation are seen in the increase in the PME peak (peak 1), and increases in the NTP peaks (peaks 4, 5, and 8). Also notable is the decrease in the NADP peak (peak 6). UDGP (peak 7) remains constant throughout the experiment. Cells proliferated during the experiment starting at 9×10^6 cells and growing to 31×10^6 cells by the 3rd day. Figure 2 show the absolute intensities of selected observable peaks and Pi/β-ATP and PME/β-ATP ratios for the experiment in Figure 1.

Experiments are currently being performed to look at the effects of estrogen antagonists on cellular proliferation in Matrigel. One such study is using the new antagonist, ICI 182,780. This drug appears to be devoid of the partial antagonist properties of tamoxifen and will be compared. Cell counts of MCF7 cells in Matrigel threads remain static over 3 day periods when cultured in media containing estradiol (1nM) and ICI 182,780 (100 nM). Figure 3 is a photomicrograph of a freshly cast MCF7 cell thread. The cells are rounded and unattached though at high density in the thread. After 48 hr incubation in media containing ICI 182,780, the cells have attached and remain viable (Figure 4) but do not proliferate as they do in media containing only estradiol. 31P NMR experiments are underway to follow intercellular phosphorus metabolism in antagonist treated cells.

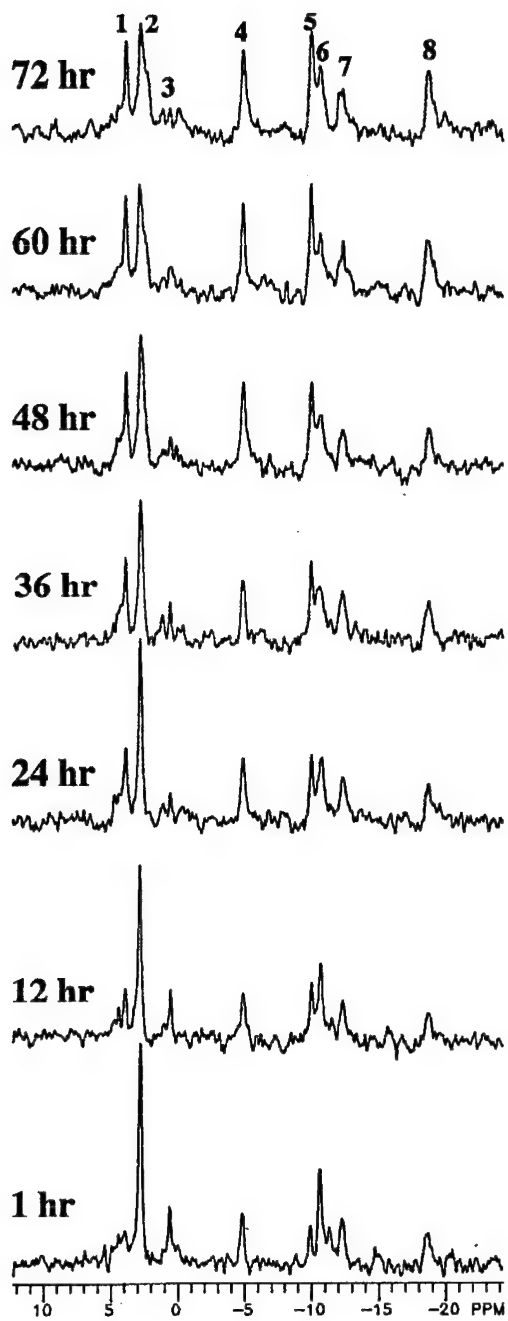

Figure 1. Spectral series of MCF7 cells proliferating in Matrigel threads over 3 day period. Peaks are phosphomonoester (1), inorganic phosphate (2), phosphodiester (3), γ-ATP (4), α-ATP (5), NADP (6), UDGP (7), and β-ATP. Cells doubled twice during 3 days of perfusion.

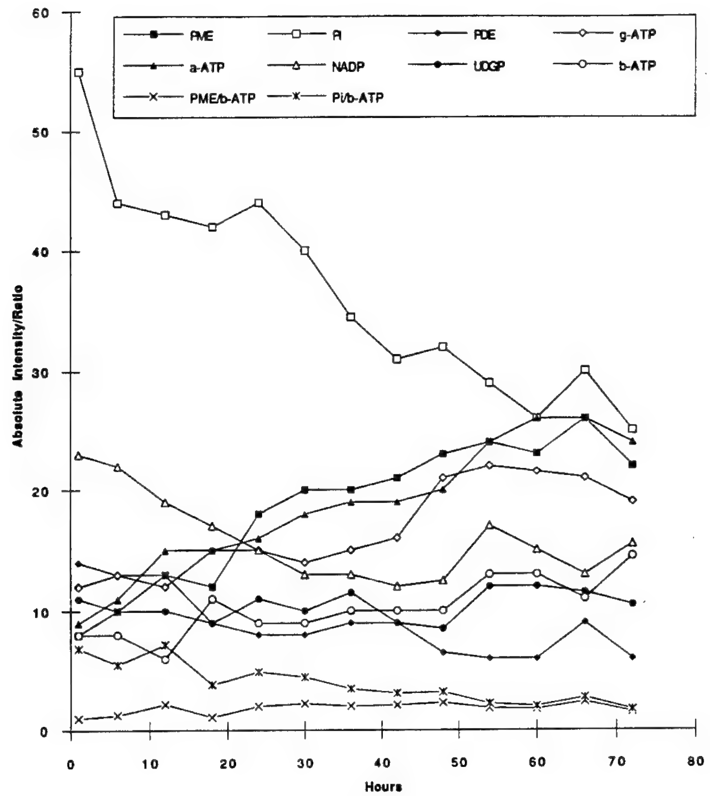

Figure 2. Absolute intensities of observed phosphorus peaks at 6 hr intervals. Consistent with cellular proliferation, increases are seen with ATP and PME peaks.

Figure 3. Photomicrograph of MCF7 cells immediately after casting into thread. Cells are rounded and not yet attached to Matrigel substrate.

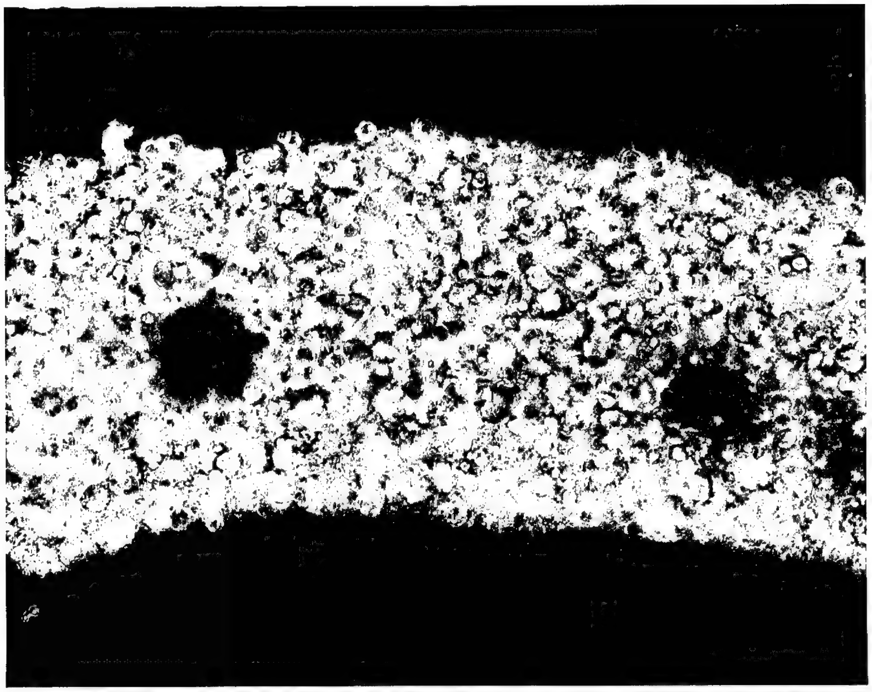

Figure 4. Photomicrograph of MCF7 cells in Matrigel thread following culturing in IMEM/CSS media containg 1 nM estadiol and 100 nM of the pure estrogen antagonist, ICI 182,780. Cells have attached and remain viable but proliferation is blocked. Dark spots are due to cell clumping

3. CONCLUSIONS

Preliminary results indicate that studies of proliferating cells can be carried out in Matrigel. Spectral studies of cellular phosphorus metabolites are consistent with cell growth when stimulated with estradiol. It appears that estrogen antagonists can reduce or block the growth of MCF7 cells cultured in Matrigel threads. NMR studies will continue with proliferating and antagonist treated cell threads. We conclude that 31P NMR may be useful for monitoring the response to hormonal treatment of breast cancer cells.

4. RECENT RELEVANT PUBLICATIONS

Ruiz-Cabello, J., Berghmans, K., Kaplan, O., Lippman, M. E., Clarke, R., and Cohen, J.S.: Hormone dependence of breast cancer cells and the effects of tamoxifen and estrogen: ^{31}P NMR studies, *Breast Cancer Res. & Treat.*, **33**:209-217, 1994.

Kaplan, O., and Cohen, J.S.: Metabolism of breast cancer cells as revealed by non-invasive magnetic resonance spectroscopy studies, *Breast Cancer Res. & Treat.* **31**:285-299, 1994.

Cohen, J.S., Jaroszewski, J., Kaplan, O., Ruiz-Cabello, J., and Collier, S.: A History of Biological Applications of NMR Spectroscopy. *Progr. Magn. Reson.* (50th anniv. edition of discovery of NMR) (J.W. Emsley and J. Feeney, eds.) Elsevier, in press, 1995.

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